REMARKS/ARGUMENTS

Status of the claims

With entry of the instant amendment, claims 5 and 7 have been cancelled. Claims 1 and 4 are therefore pending and under examination.

Cancellation of subject matter is without prejudice to subsequent revival for prosecution in a continuation or divisional application.

The objections/rejections will be addressed in the order set forth in the Office Action mailed March 21, 2007.

Objection to the specification

The objection to the specification has been obviated by the amendment.

Rejection under 35 U.S.C. § 101, utility

Claims 1, 4, 5, and 7 are rejected as allegedly lacking utility. The Examiner contends that the specification fails to assert a specific and substantial utility. The Examiner does not dispute that the transgenic animals described in the specification exhibit a phenotype. The Examiner alleges, however, that an artisan would not find the asserted utilities to be specific or substantial because the specification, according to the Examiner, does not provide a correlation between a melanopsin gene and an established function, phenotype or disease. In the interests of expediting prosecution, claims 5 and 7 have been cancelled. With regard to claims 1 and 4, Applicants respectfully traverse.

The claimed transgenic animals have utility for the evaluation of candidate compounds that modulate circadian clocks.

The Examiner contends that there is no nexus between melanopsin and the various disorders listed in paragraph 54. However, this positions overlooks the totality of the teachings in the specification. The eye is the principal mediator of light input to the central nervous system in mammals (*see, e.g.,* paragraph 104). The communication of light information

provides important temporal information to the circadian clock (*e.g.*, paragraphs 4-6). As the Examiner has noted, the specification in fact discloses a phenotype of melanopsin knockout animals: *e.g.*, the claimed mice exhibit attenuation in phase shifting relative to wildtype control animals. The specification further teaches that the attenuation in phase shifting in the claimed animals is a direct result of reduced sensitivity of the photic input pathway to light (*e.g.*, paragraph 102). The specification therefore establishes a role of melanopsin in light input. In view of the role light information plays in circadian control, there is a nexus between various disorders of the circadian clock (*e.g.*, paragraphs 7 and 54) and melanopsin.

One of skill would reasonably believe the asserted utility is true

The Examiner alleges that the phenotype exhibited by melanopsin knockout animals is insufficient such that one of skill would know how to the use of melanopsin knockout animals for the evaluation of compounds that modulate circadian clocks. The Examiner is respectfully reminded that applicant need not provide evidence that establishes that an asserted utility is a statistical certainty. Instead, evidence is sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true (MPEP §2170.02.VII). Here, the evidence as a whole suggests that one of skill would be more likely than not to believe that melanopsin knockout mice would be useful for evaluating the effects of compounds on light sensitivity of the photic input pathway.

The Examiner cites Beaule *et al,* in *J. Mol. Neurosci* 21:73-89, 2003 ("Beaule") as evidence in support of the position that the asserted utility would not be recognized by one of skill in the art. In particular, the Examiner cites Beaule as allegedly providing evidence that melanopsin is not, in and of itself, necessary for circadian photoreception. The Examiner contends that the invention is therefore not useful because other genes may also play a role in photoreception and circadian clocks. The observation that other gene may also play a role in modulating light input, however, does not negate the utility of the claimed invention. Indeed, Beaule does not dispute that melanopsin plays a role in sensitivity of mammals to light exposure. Beaule states that the transgenic animals studies suggest that melanopsin plays a role in mediating the acute effects of light and the disruptive effects of constant light on circadian

rhythms (second column of page 75 bridging to the first column of page 76). Thus, the teachings of Beale in fact support Applicants' assertion that melanopsin plays a role in mediating light effects on the circadian clock.

The Examiner also cites Kumbalasiri *et al, in Exp. Eye Res.* 81:268-375, 2005 ("Kubalasiri") as suggesting that there is no nexus between melanopsin and circadian disorders because other opsins may also be involved in the transgenic mouse phenotype. However, Kumbalasiri again supports the teachings of the specification relating to the role of melanopsin in photic input pathway sensitivity to light. For example, on page 371 (second column, last paragraph), Kumbalasiri explains that studies of melanopsin-null mice show that melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) play a role in adjustment of circadian phase in response to light pulses, regulation of circadian period in response to constant light, and papillary light reflex as well as other light sensing photo pathways.

The Examiner specifically cites Kumbalasiri in support of the position that the claimed animals would not be useful because compensation by other opsins could occur. Applicants again respectfully disagree. First, Kumbalasiri does not suggest that other opsins could compensate for melanopsin. Further, there is no evidence that such compensation exists for melanopsin, and even assuming *arguendo* that it occurs at all, renders the claimed knockout animals useless. Applicants have established that melanopsin-null mice are impaired in light-induced phase delay responses and period lengthening of the circadian rhythm in constant light. Other investigators have independently confirmed this finding, as explained in Applicants' response of the previous Office Action. Thus, one of skill could reasonably expect to obtain this phenotype.

Furthermore, experiments in the specification using melanopsin knockout animals that are also lacking functional rods and codes exhibited a completed loss of photic responses (see, *e.g.*, paragraph 119), providing additional evidence that inactivating melanopsin leads to an observable phenotype that is not compensated for by other non-visual opsins. This is further confirmed by Hattar *et al.* in *Nature* 424:76-81, 2003 ("Hattar", attached in Appendix A). Hattar confirms the understanding in the art that melanopsin knockout mice have attenuation of light-induced phase delays in the circadian clock and exhibit period lengthening of the circadian

rhythm in constant light. Hattar then teaches that animals lacking melanopsin and having disabled rod and cone transduction fail to show any significant pupil reflex, to entrain to light/dark cycles and to show any masking response to light. Hattar concludes that the melanopsin and rod-cone systems together seem to provide all of the photic input for these accessory visual functions (see, *e.g.* the last two sentence in column 1, page 76, bridging to column 2).

Thus, in view of the foregoing, the phenotype exhibited by the claimed mice reflect a biological role that would lead one of skill to more likely than not, believe the utility of the invention for evaluating test compounds and their effect on circadian clock disorders.

The claimed subject matter has specific and substantial utility in a laboratory setting

Melanopsin knockout mice also have utility in a laboratory setting as a research tool for studies involving sensitivity of photic pathways to light. The MPEP explicitly cautions against labeling inventions as lacking specific and substantial utility based on the setting, such as a laboratory, in which the invention is to be used.

> Some confusion can results when one attempts to label certain types of inventions as not being capable of having a specific and substantial utility based on the setting in which the invention is to be used. One example is inventions to be used in a research or laboratory setting. Many research tools... have a clear, specific and unquestionable utility.... An assessment that focuses on whether an invention is useful only in a research setting...does not address whether the invention is in fact "useful" in a patent sense. (MPEP § 2107.01)

In the case of melanopsin knockout mice, utility in a laboratory setting is credible, specific, and substantial. First, a utility is specific if it is not applicable to a broad category in general (MPEP § 2107.01.I). Here, melanopsin plays a specific role in photic input. This role is not only supported in the specification, but further confirmed by other investigators, as explained above. This function is not applicable to a broad category, *i.e.*, proteins, or even other opsins, in general. Accordingly, the utility is specific to the subject matter claimed.

The use of melanopsin transgenic animals in a laboratory setting is also a substantial utility. The MPEP (§2107) defines a substantial utility as a real world use. The

claimed animals have a practical, real-world use. Indeed, the literature cited by the Examiner shows that photic input and circadian control is an active area of investigation, and hence, provides a practical and specific context for using the claimed transgenic knockout animals in a laboratory setting.

Last, this is a credible utility. As noted above, in order to find to the contrary, the Office personnel would have to establish that it would be more likely than not that one of ordinary skill in the art would doubt the truth of the utility. (MPEP § 2107.02.III.A). Although the Examiner cites Beaule and Kumbalasiri in support of the position that one of skill would not believe the role of melanopsin in circadian control, the totality of the teachings of this reference point to a role in circadian control. Moreover, the specification teaches that melanopsin plays a role in the response to light. This role is additionally acknowledged in the post-filing art of record. An ordinary artisan would thus believe that the utility of melanopsin knockout animals in a laboratory setting is real.

In view of the foregoing, the invention meets the requirements for utility. Applicants therefore respectfully request withdrawal of the utility rejection under 35 U.S.C. § 101 and its associated rejection under 35 U.S.C. § 112, first paragraph.

Rejection under 35 U.S.C. § 112, first paragraph--enablement

The claims were also separately rejected for alleged lack of enablement. In the interests of expediting prosecution, claims 5 and 7 have been cancelled. The rejection will therefore be addressed as it has been applied to claims 1 and 4. First, on page 9 of the Office Action, the Examiner contends that claims 1 and 4 do not recite any specific phenotype associated with the transgenic knockout mouse. However, this is incorrect. Claim 1 recites a mouse that exhibits an attenuated circadian rhythm phase-shift in response to a light pulse during a dark portion of an environmental dark/light cycle.

In the rejection and response to Applicants' arguments submitted in response to the previous office action, the Examiner again alleges that because there is no specific phenotype linking a melanopsin knockout mouse to a specific condition or disease, one of skill would not know whether the attenuation of the phase shift described in the specification is due to

melanopsin gene knockout or because of compensatory factors. The Examiner therefore concludes that the artisan would have to perform undue experimentation to make use of the invention. The Examiner further asserts that it would require undue experimentation to determine if the symptoms associated with the knockouts animals are representative of a disease. Again, Applicants respectfully traverse. The specification unambiguously demonstrates that melanopsin plays a role in light-sensitive photic input pathways and that one of skill could generate and use the claimed melanopsin-null mice.

The Examiner again cites to Beale as disclosing that results from transgenic knockout experiments do not provide support for a critical role of melanopsin in normal photic entrainment. However, Beaule states that the results show that melanopsin in and of itself does not appear to pay a critical role. This does not mean that melanopsin plays no role. Indeed, as explained above, melanopsin undoubtedly plays a role.

The Examiner also points to various other references (referred on paged 10 and 14 of the office action) in support for the assertion that the effects of deletion of a given depends on the genetic background However, as previously expained, art of record (see, *e.g.,* Applicants' response to the previous Office Action) demonstrate that melanopsin affects the ability to shift the phase of circadian activity rhythms in response to light pulses. This is further acknowledged by Kumbalsiri (page 372, first column first full sentence). Thus, more than one transgenic mouse model has exhibited a phenotype that has been described by Applicants. Therefore, although phenotypes may be influenced by genetic background, here, the art as a whole suggests that a melanopsin knockout animal with a phenotype relating to its role in photic input can reasonably be expected to be obtained by a practitioner.

The Examiner also alleges that potential compensation by other opsins would prevent a practitioner from successfully using the invention without undue experimentation. However, again, as the Examiner has acknowledged, melanopsin animals have a defined phenotype. This is reproducible regardless of any (theoretical) compensation that may be occurring due to other opsins.

In view of the foregoing, one of skill in the art could reasonably expect to be able to generate animals with a phenotype as set forth in the claims and to be able to use such

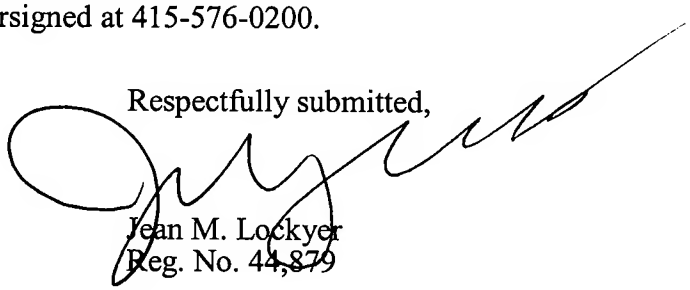
melanopsin-null knockout animals without undue experimentation. Accordingly, the claims are properly enabled. Applicants therefore respectfully request withdrawal of the rejection.

CONCLUSION

Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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APPENDIX A

Melanopsin and rod–cone photoreceptive systems account for all major accessory visual functions in mice

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In the mammalian retina, besides the conventional rod–cone system, a melanopsin-associated photoreceptive system exists that conveys photic information for accessory visual functions such as pupillary light reflex and circadian photo-entrainment[1–7]. On ablation of the melanopsin gene, retinal ganglion cells that normally express melanopsin are no longer intrinsically photosensitive[8]. Furthermore, pupil reflex[8], light-induced phase delays of the circadian clock[9,10] and period lengthening of the circadian rhythm in constant light[9,10] are all partially impaired. Here, we investigated whether additional photoreceptive systems participate in these responses. Using mice lacking rods and cones, we measured the action spectrum for phase-shifting the circadian rhythm of locomotor behaviour. This spectrum matches that for the pupillary light reflex in mice of the same genotype[11], and that for the intrinsic photosensitivity of the melanopsin-expressing retinal ganglion cells[7]. We have also generated mice lacking melanopsin coupled with disabled rod and cone phototransduction mechanisms. These animals have an intact retina but fail to show any significant pupil reflex, to entrain to light/dark cycles, and to show any masking response to light. Thus, the rod–cone and melanopsin systems together seem to provide all of the photic input for these accessory visual functions.

Rods and cones have long been thought to be the exclusive photoreceptors in the retina. This hypothesis is now known to be untrue. An opsin-like protein called melanopsin, originally identified in *Xenopus* skin melanophores[1], is present in a small subset of mammalian retinal ganglion cells (RGCs)[1–6], and these cells are intrinsically photosensitive[6,7]. The axons of these RGCs project predominantly to the suprachiasmatic nucleus (SCN), the intergeniculate leaflet (IGL) and the olivary pretectal nucleus (OPN) of the brain[6], which are key centres for circadian photo-entrainment and the pupillary light reflex. In melanopsin knockout mice ($Opn4^{-/-}$, formerly referred to as $mop^{-/-}$; see ref. 8), those RGCs that would normally express melanopsin lose their intrinsic photosensitivity[8]. $Opn4^{-/-}$ mice also have an incomplete pupillary light reflex at high illuminations[8]. In independently produced melanopsin-knockout mice, others have found that the ability of light to phase-delay and lengthen the period of the circadian rhythm is also diminished[9,10]. For the pupil reflex, this photic response can be quantitatively accounted for by a functional complementarity between the rod–cone system and the melanopsin system, without the need to invoke any additional light-detection system[8]. Nonetheless, the proposal has persisted that cryptochromes—flavoproteins reported to have a direct light-detecting role in *Drosophila*[12,13]—may have the same function in mammals[14–16] despite earlier evidence to the contrary[17]. To settle this question, we first examined the action spectrum for phase-shifting the circadian rhythm in mice lacking rod and cone photoreceptors (*rd/rd cl*)[18]. Next, we generated triple-knockout mice lacking all confirmed photodetection systems—$Opn4^{-/-}$ $Gnat1^{-/-}$ $Cnga3^{-/-}$ (melanopsin (also known as opsin 4), guanine nucleotide-binding protein α-transducin 1 (also known as rod transducin α-subunit, or Trα) and cyclic GMP-gated channel A-subunit 3, respectively)—and tested these animals for pupil reflex, circadian photo-entrainment and the masking response to light.

Irradiance–response relations for the light-induced phase shifting of the circadian rhythm of locomotor behaviour in *rd/rd cl* mice were measured at various wavelengths (Fig. 1a). The irradiance for half-maximal phase shift at each wavelength was then plotted to give the action spectrum (Fig. 1b). This spectrum is best fitted by the predicted absorption spectrum of a vitamin A_1-based photopigment with a wavelength of maximum absorbance (λ_{max}) ≈ 481 nm, similar to that for the pupil reflex in this genotype (λ_{max} ≈ 479 nm)[11] and, more specifically, that for the intrinsically photosensitive, melanopsin-expressing RGCs in the rat (λ_{max} ≈ 484 nm)[7]. Thus in the absence of rods and cones, circadian photo-entrainment is apparently determined by the melanopsin system. Previously, an action spectrum with a λ_{max} of 480 nm has been reported for

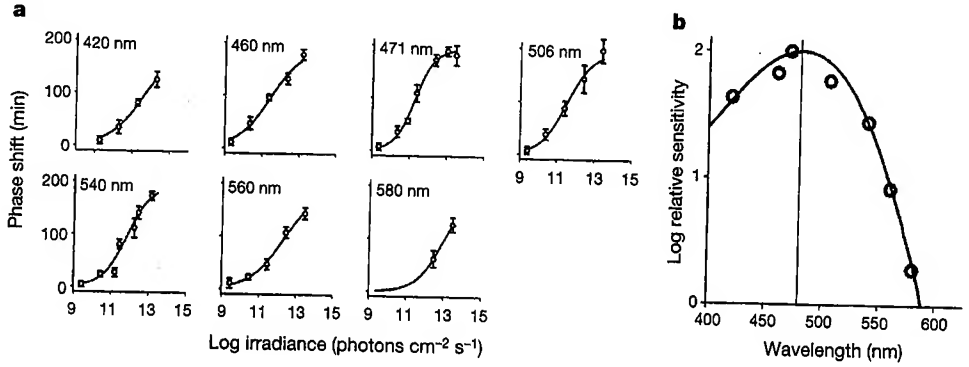

Figure 1 Irradiance–response relations (**a**) and action spectrum (**b**) for circadian phase shifting in *rd/rd cl* mice by monochromatic light between 420 nm and 580 nm, assayed by wheel-running (n = 4–7 animals per irradiance at each wavelength). The derived action spectrum in **b** for circadian photo-entrainment is best fitted (R^2 = 0.976) by the absorption spectrum of a vitamin A_1-based photopigment with λ_{max} = 481 nm (continuous curve).

circadian phase shifting by light in one strain of *rd* mice that has lost rods and perhaps most cones[31]. However, in part because the same experiments on a different strain of *rd* mice gave a λ_{max} between 500 nm and 515 nm (ref. 32) and interpreted to reflect the action spectrum of mouse middle-wavelength-sensitive (M)-cones, the significance of the first study was never settled. With the *rd/rd cl* mouse line described here in which the loss of rods and cones is essentially complete, however, the signal from residual cones is no longer an issue.

To obtain more conclusive evidence that no other independent photoreceptive systems exist for the various accessory visual functions, we generated triple-knockout mice in which the rod–cone system and the melanopsin system were both silenced. Notably, the silencing of rod–cone functions in this case was achieved not by inducing degeneration of these cells (as occurs in *rd/rd cl* mice) but by combining targeted deletions of the genes for rod *Gnat1* (ref. 19) and cone *Cnga3* (refs 20, 21). Gnat1 and Cnga3 are critically involved in the G-protein-coupled cGMP signalling pathway that mediates rod–cone phototransductions. Therefore, in triple-knockout mice, the melanopsin system and the rod–cone system are both unable to signal light. We investigated whether these animals still had any residual response to light, as assayed by their pupil reflex and locomotor behaviour.

The *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$ mice were generated by first producing triple heterozygotes (*Opn4*$^{+/-}$ *Gnat1*$^{+/-}$ *Cnga3*$^{+/-}$) and then mating these to homozygosity. These mice had a normal-looking retina (Fig. 2a). Because *Opn4*$^{-/-}$ was produced by replacing the melanopsin gene with the tau-LacZ construct[6,8], the RGCs that would normally express melanopsin could be visualized by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) labelling.

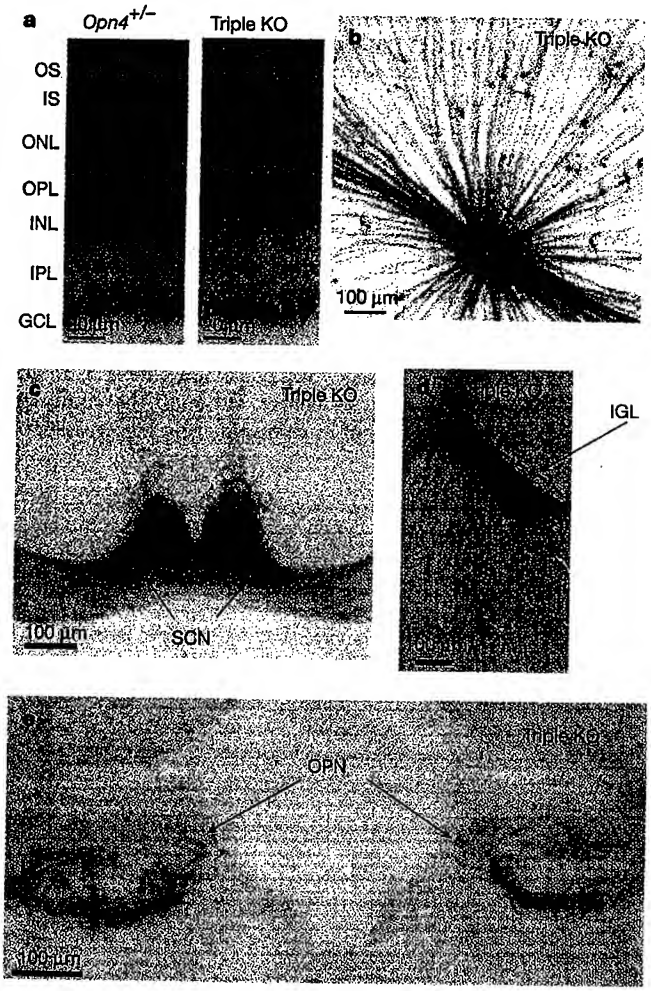

Figure 2 Normal retinal morphology and presence and central connectivity of melanopsin-expressing RGCs in triple-knockout (*Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$) mice. **a**, Retinal cross-sections from *Opn4*$^{+/-}$ and triple-knockout (KO) mice. Giemsa stain shows the various layers. Both are similar in morphology and thickness to wild type. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment layer. **b**, Flat-mount view of a triple-knockout retina stained with X-gal (blue). **c**–**e**, Coronal sections of triple-knockout mouse brain showing the normal innervations of the SCN, IGL and OPN by X-gal-labelled axons. Dorsal side is up in each case.

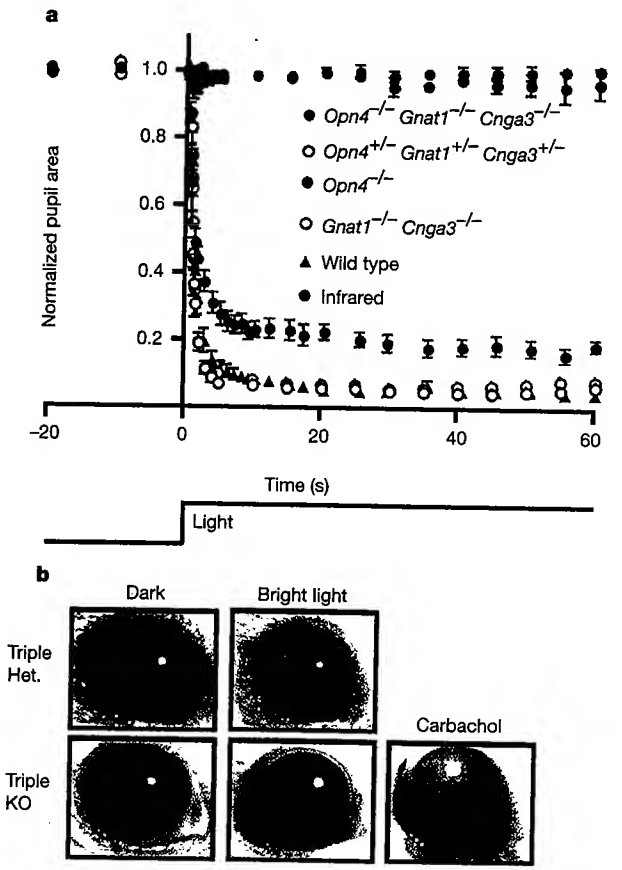

Figure 3 Disabling of rods, cones and melanopsin-positive RGCs essentially eliminates the pupillary light reflex. **a**, Pupil area (mean ± s.e.m.) as a percentage of dark-adapted aperture area (recorded just before light exposure, time 0 on graph) for *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$ ($n = 6$), *Opn4*$^{+/-}$ *Gnat1*$^{+/-}$ *Cnga3*$^{+/-}$ ($n = 4$), *Opn4*$^{-/-}$ ($n = 8$; data reproduced from ref. 8) and *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$ ($n = 1$) mice over the course of 1 min when exposed to 480-nm light at 86–140 μW cm^{-2}. The wild-type data are from ref. 8. For comparison, the pupil size of triple-knockout mice exposed to an infrared light pulse ($n = 6$) is also shown. On close scrutiny, a small, transient pupil constriction was detected in two out of six triple-knockout mice exposed to the intense 480-nm stimulus. The averaged 480-nm data shown for the triple knockouts are from mice dark-adapted for 3 days before light exposure, but are representative of similar recordings from the same group after 1-h dark adaptation. Neither the amplitude nor the frequency of occurrence of the residual pupillary response in the triple-knockout animals were increased with bright white-light stimulus (30 mW cm^{-2}), or with monochromatic stimuli ranging from 360 to 660 nm (70 μW cm^{-2} at 360 nm; 19 μW cm^{-2} at 420 nm; 86 μW cm^{-2} at 480 nm; 100 μW cm^{-2} at 550 nm; 68 μW cm^{-2} at 660 nm). Nor did these parameters show any significant change when tested repeatedly over a 24-h period. **b**, Application of 100-mM carbachol resulted in a complete pupil constriction in triple-knockout mice.

In the triple-knockout mice, these cells were still present (Fig. 2b) in numbers (about 600 per retina) comparable to wild type[6,8], and their axons still projected predominantly to the SCN, IGL and OPN of the brain (Fig. 2c–e). Thus, the absence of melanopsin and of functional rod–cone phototransductions does not affect the genesis, survival and central connectivity of the melanopsin-associated RGCs.

When tested for the pupillary light reflex with steady, intense exposure of light at 480 nm, the triple-heterozygous mice gave a response resembling wild type in amplitude and time course (Fig. 3a). The double-knockout mice (*Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$) showed the same response (Fig. 3a), similar to what we found previously[11] for *rd/rd cl* mice, which have complete degeneration of rods and cones. Triple knockouts, however, hardly gave any response to the 480-nm stimulus (Fig. 3a), nor to monochromatic light at other wavelengths (360–660 nm) or intense white light (data not shown). The same general results were obtained when these animals were tested at various times over a 24-h period. On close inspection, there was a very transient, barely detectable pupil response (with a mean of 5% reduction in pupil area at peak response) in two out of six tested triple-knockout mice under exposure to bright 480-nm light. However, even in animals where this residual response was detected, it was not consistently present on repeated stimulus trials with extensive dark adaptation (up to 3 days) in between (see Fig. 3 legend). The response was unlikely to be caused by heat associated with the illumination, because it largely disappeared after replacement of the 480-nm interference filter with a band-pass filter transmitting only infrared light (≥850 nm) (Fig. 3a). A possible source of the signal is the early receptor potential of rods and cones, which consists of a very small, transient membrane hyperpolarization caused by charge movements associated with conformational changes of the visual pigments after photo-isomerization[22,23]. This hyperpolarization should persist regardless of whether the transduction steps downstream of the pigment are disabled or not. Another possibility is a very small, transient hyperpolarization generated by intense light in rods and cones via a pathway apparently independent of transducin[24]. In any case, the smallness of the residual pupil response, its transient nature even in steady, intense light, and the inconsistency of its occurrence all suggest that it is unlikely to be of physiological significance. Finally, the failure of the *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$ pupil to constrict was not due to a defect in the constriction mechanism because carbachol, a parasympathetic agonist, was able to activate maximum pupil constriction when applied to the cornea (Fig. 3b).

To assess photo-entrainment, *Opn4*$^{+/-}$ *Gnat1*$^{+/-}$ *Cnga3*$^{+/-}$ and *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$ animals separate from those used in the pupil-reflex studies were kept in an 16/8-h light/dark cycle (800 lx white light in the light period), and their locomotor activity was monitored (see Methods). The triple-heterozygous mice showed normal photo-entrainment, whereas the triple-knockout mice did not show any entrainment (Fig. 4; see also Supplementary Information). Actograms indicated that the triple-heterozygous mice had an average period length of 24.0 ± 0 h (mean ± s.e.m., $n = 6$; individually all 24.0 h), as expected from stable photo-entrainment. In contrast, the triple homozygous mice had a period length of 23.3 ± 0.2 h ($n = 5$; individually 23.8, 23.3, 23.2, 22.5 and 23.5 h), similar to the 23.6 h reported for the same strain (B6/129) of wild-type mice in constant darkness[25], indicating that these animals free-ran even under light/dark conditions. Of note, at the light intensity used in these experiments, the pupils of wild-type or triple-heterozygous mice would have constricted considerably (see Fig. 3a), whereas those of the triple-knockout mice would have stayed unchanged. Thus, the equivalent light intensity at which the triple-knockout mice failed to show photo-entrainment was actually much higher than that capable of entraining wild-type or triple-heterozygous mice.

We have also examined another accessory visual function, namely the masking of locomotion of nocturnal rodents by light, which involves a fast and direct effect of light independent of the circadian pacemaker[26–28]. To examine masking by light, the mice tested for photo-entrainment were subsequently placed on a 3.5/3.5-h light/dark cycle (800 lx white light as before, although the equivalent intensity for the triple-knockout mice would again be considerably

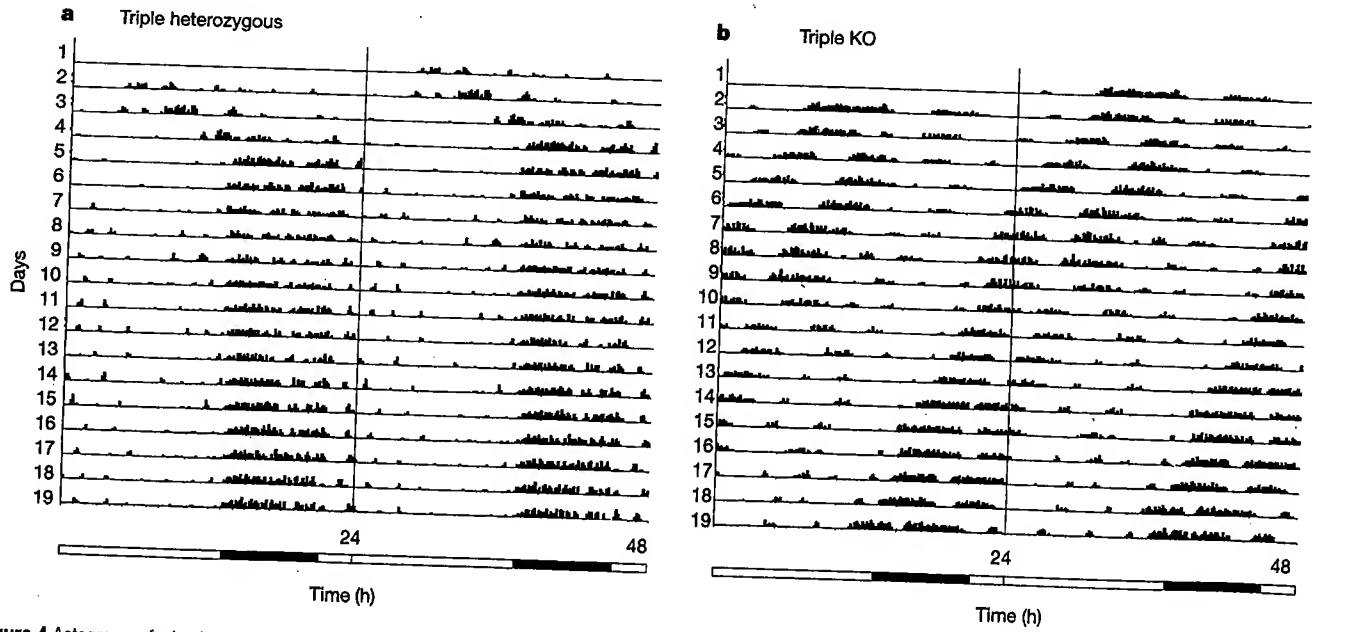

Figure 4 Actograms of wheel running for mice under a 16/8-h light/dark cycle, double-plotted on a 24-h timescale. The illumination was approximately 800 lx white light. The numbered lines represent successive days. Activity levels (in total number of revolutions in 10-min bins) are given in 15 quantiles, with the first being 1–55 revolutions, the second 56–110, and so on. The bar below the actograms indicates light (white) and dark (black) periods. **a**, Triple heterozygote (*Opn4*$^{+/-}$ *Gnat1*$^{+/-}$ *Cnga3*$^{+/-}$). The locomotor activity had a cycle very close to 24 h and was phase-locked to darkness, showing photo-entrainment. **b**, Triple-knockout (*Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$). The animal free-ran with a period of less than 24 h, showing lack of photo-entrainment.

higher than for the triple-heterozygous mice when the lack of pupil reflex in the triple-knockout animals is taken into account). This ultradian cycle is useful for assessing masking because it is difficult to entrain circadian rhythms to cycles with periods of 7 h or multiples thereof. In this way, it was ensured that masking could be measured at different phases of the endogenous clock[29]. When kept in this cycle, the triple-heterozygous mice showed two effects. Four of the six tested animals were negatively masked (that is, their locomotor activity was diminished) by light (Fig. 5a). The average percentage of activity in the dark period for these 'light avoiders' was 87.1 ± 8.8% (mean ± s.e.m.; individually 99.5%, 66.3%, 85.0% and 97.7%), similar to what was seen with B6/129 wild-type mice ($n = 12$; N.M. and S.H., unpublished data). The remaining two animals, paradoxically, developed more wheel-running activity in the light period (Fig. 5b), possibly due to the presence of the 129-strain background associated with the triple-heterozygous genotype (this activity reversal has previously been observed in other mouse lines with the 129-strain background; R.J.L., unpublished data). Although the reason for this reversal of activity with respect to the light/dark cycle is unclear, undoubtedly light still had an effect (in this case, positive masking). The percentage of activity in the dark period for these two 'light preferers' was 32.7% (Fig. 5b) and 26.0%, respectively. The triple-knockout mice, on the other hand, were hardly affected by this ultradian light/dark cycle (Fig. 5c, d). The average percentage of activity in the dark period was 43.2 ± 3.4% (mean ± s.e.m., $n = 4$; individually 44.7%, 50.5%, 40.8% and 36.6%), much closer to that expected from randomness (50%).

Given the fact that RGCs normally expressing melanopsin still project to the appropriate brain targets in triple-knockout mice, the conclusion from our experiments is that the rod–cone system and the melanopsin system are the exclusive light-detecting systems in the eye for producing the normal pupillary light reflex, photoentrainment and masking response to light. These experiments go beyond our previous conclusion that the rod–cone and melanopsin systems are fully complementary to each other in the pupil reflex[8], by demonstrating an essentially complete loss of this and other functions when the two systems are absent or disabled. It was recently reported[16] that the pupil reflex of mice lacking rods as well as Cry1 and Cry2 (*rd/rd Cry1*$^{-/-}$ *Cry2*$^{-/-}$) is 100-fold less photosensitive than normal (and 10-fold less sensitive than mice only lacking rods; *rd/rd*), proposed to support the idea that the cryptochromes may participate as a photodetective system. Nonetheless, other interpretations of these earlier data are possible. Furthermore, the pupil reflex of mice only lacking Cry1 and Cry2 is normal (as is their masking response to light[30]). A potential complicating factor in interpreting the results from mice lacking Cry1 and Cry2 is that these animals are reported to show frequent ocular inflammation[15], which may cause subtle changes in retinal function. In any case, we know from immunocytochemistry of our *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$ mice that at least Cry2 is still widely expressed in the inner retina (including the RGCs expressing melanopsin), comparable to wild type (data not shown)—the antibodies for Cry1 did not give informative labelling; see Methods. At the same time, real-time polymerase chain reaction with reverse transcription (RT–PCR) indicated that the message levels for Cry1

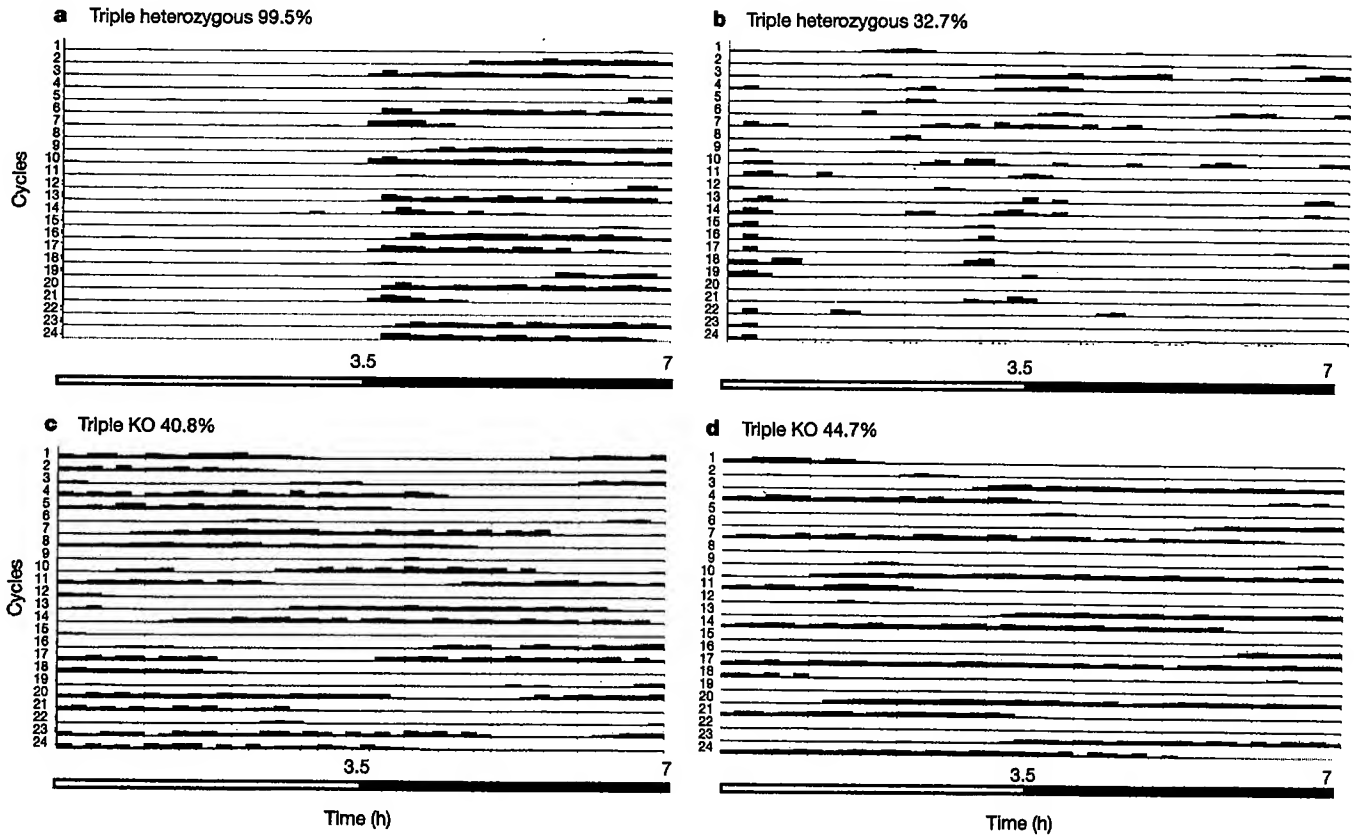

Figure 5 Actograms of wheel running plotted on a 7-h timescale. The mice were subjected to an ultradian 3.5/3.5-h light/dark cycle (see text). Illumination was approximately 800 lx white light. The numbered lines represent successive 7-h cycles. The activity quantiles and time bins are the same as in Fig. 4. **a**, **b**, Two triple-heterozygous mice (*Opn4*$^{+/-}$ *Gnat1*$^{+/-}$ *Cnga3*$^{+/-}$) showing a negative and positive masking effect, respectively. The percentage of total revolutions in darkness was 99.5% and 32.7%, respectively. **b**, Two triple-knockout mice (*Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$). The percentage of total revolutions in darkness was 40.8% and 44.7%, respectively, not far from the 50% expected from randomness; that is, no masking effect of light.

and Cry2 in *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ *Cnga3*$^{-/-}$ eyes are comparable to those in wild-type mice (Supplementary Information). Thus, the disappearance of the accessory visual functions in the triple-knockout mice cannot be attributed to secondary loss of cryptochromes. Even if mammalian cryptochromes should turn out to function as direct light detectors, either they obligatorily depend on the presence of melanopsin for their light-detecting function or they do not signal light at least for the diverse photic responses studied here. □

Methods

Animals

The *rd/rd cl* mice were bred at Imperial College London, and the circadian phase-shifting experiments were carried out there. The triple-knockout and the corresponding triple-heterozygous mice were bred at Johns Hopkins. The pupil reflex experiments were done at Hopkins, whereas the wheel-running experiments were done at the University of Toronto. At the time of experimentation, the Hopkins animals were 1–4 months old for the pupil reflex measurements and 1–9 months old (1–9 months for triple-heterozygous mice, 1–2 months for triple-knockout mice; all were males except for one female triple knockout, which was used for photo-entrainment but not for masking experiments) for the wheel-running measurements. For morphological, X-gal labelling, immunocytochemical and RT–PCR experiments, the animals were 2–3 months old.

Giemsa staining of retinal cross-sections

An animal anaesthetized by intraperitoneal injection of avertin (0.2 ml g^{-1}) was circulationally perfused with 25 ml cold 4% paraformaldehyde in PBS for 15 min. The eyes were isolated, corneas removed, and fixed for an additional 3 h in cold 4% paraformaldehyde in PBS. The eyes were cryo-protected overnight in 30% sucrose in PBS at 4 °C, then sectioned at 10 μm thickness on a cryostat. The sections were allowed to dry and stained with the nucleus-labelling Giemsa solution (Sigma).

Assays for Cry1 and Cry2 in triple-knockout eyes

We used two assays. The first was immunocytochemistry[6], with antibodies PA1-527 (Affinity Bioreagents) and CRY11-A (Alpha Diagnostic International) against Cry1, and CRY21-A (Alpha Diagnostic International) against Cry2. The two antibodies against Cry1 did not give specific labelling in the retina, but Cry2 gave labelling specific enough for comparison with wild-type retina. The second assay was with RT–PCR using Cry1- and Cry2-specific primers. The samples were whole eyes without the lens, and RNA was extracted using the trizol method (Sigma). The controls were RNA samples without reverse transcriptase. Real-time RT–PCR was carried out with SYBR green detection on an ABI Prism 7700 machine (Applied Biosystems). The identity of the product was checked by melting-curve analysis, size confirmation on an agarose gel and sequencing of the products.

X-gal staining

For X-gal labelling of the brain sections or flat-mount retina of the triple-knockout mice, an animal was anaesthetized and circulationally perfused with fixative as above, and the brain/eyes were isolated. An eye-cup was prepared by removing the anterior half of the eye, while the brain was frozen on dry ice and sectioned at 50-μm thickness with a sliding microtome (Leica SM2000R). Brain sections were mounted on microscope slides (Fisherbrand, superfrost-plus) and allowed to dry overnight. To stain for β-galactosidase activity, both preparations were washed twice in buffer B (100 mM phosphate buffer, pH 7.4, 2 mM $MgCl_2$, 0.01% Na-desoxycholate and 0.02% (octylphenoxy) polyethoxyethanol (IGEPAL)), and then incubated in the staining solution (buffer B plus 5 mM K-ferricyanide, 5 mM K-ferrocyanide and 1 mg ml^{-1} X-gal) for 6–10 h at room temperature in darkness. Afterwards, the retina was isolated from the eye-cup and flat mounted. Both brain sections and the retina were mounted in glycerol. Images were obtained using a Zeiss Axiophot microscope and AxioCam HRc digital camera.

Phase shifting of locomotion in *rd/rd cl* mice

Singly housed male C3H/He *rd/rd cl* mice aged between 80 and 250 days were maintained in constant darkness. A single 15-min monochromatic light pulse (half bandwidth = 10 nm) of defined irradiance was applied after 7 days at circadian time 16 (4 h after activity onset) at 420, 460, 471, 506, 540, 560 and 580 nm, respectively. To correct for lens transmission at different wavelengths, lenses were extracted from *rd/rd cl* mice ($n = 4$) and wavelength-scanned in air using an integrating sphere (see Supplementary Information). The irradiances indicated have been corrected for lens transmission. The magnitude of phase shift in free-running locomotor activity rhythm by light at each wavelength was plotted against irradiance and fitted to a sigmoidal function. The irradiances that produced half-maximum phase shifts were plotted against wavelength to produce the action spectrum. This spectrum was fitted with the absorption spectrum of a vitamin A_1-based pigment with a best-fit λ_{max} determined by least squares.

Pupillometry

Consensual pupillary constriction was measured in response to an adirectional light stimulus. Light was provided by a PTI xenon arc-lamp, filtered to remove wavelengths <299 nm and most infrared, and transmitted by means of a liquid light guide to a diffusing sphere. This sphere was constructed from a ping-pong ball and designed to render the light stimulus nearly adirectional at the location of the mouse cornea. An adult mouse, dark-adapted for 1 h and unanaesthetized, was positioned and oriented with the help of an infrared image-converter such that one eye was situated at the exit of the reflective sphere and the other eye was in focus, and oriented as perpendicular as possible, to a charge-coupled device (CCD) camera fitted with a × 10 macro lens. Illumination of the videoed eye was with infrared LEDs (>850 nm) throughout the experiment. Pupil measurements from the video images of the eye were made using TINA 3.1 image-analysis software and a Matrox meteor-II frame-grabber card (Matrox Imaging). The intensity and wavelength of the light stimulus were controlled with calibrated neutral-density and interference (10-nm bandwidth) filters. Irradiance measurements (W cm^{-2}) were made with a radiometer (Macam Photometrics). Unless indicated otherwise, all experiments reported here were conducted during the light period of a 14/10-h light/dark cycle (between 9 and 3 h before lights off)[8].

Carbachol application

Carbachol (carbamylcholine chloride) was obtained from Sigma. Solution (100 mM) was prepared in × 1 PBS, pH 7.4, and filtered through a 0.22-μm filter. Samples were topically applied to the cornea of the mouse eye with a microtip transfer pipette. The pupil size in darkness was assessed immediately before and at 5 min after carbachol application. We found that a carbachol concentration of 100 mM was able to fully constrict the eyes of both triple-heterozygous and triple-knockout mice, and therefore we did not use higher concentrations (see ref. 8).

Photo-entrainment and masking experiments

The mice were placed individually in cages (44 × 23 × 20 cm) equipped with running wheels (17.5 cm in diameter). Room temperature was 19.5–23.5 °C. Revolutions were recorded with Dataquest III hardware and software in 10-min bins. The light/dark cycle was 16/8-h light/dark or 3.5/3.5-h light/dark, with approximately 800 lx in the light period (Hagner EX2 luxmeter) illuminated by Sylvania Octron 32 W 4100K fluorescent tubes. For photo-entrainment, the animals were studied for 19 days on the 16/8-h light/dark cycle, with data from the last 10 days used for period analysis based on the algorithm of Clocklab (Actimetrics) for the Bushell and Sokolove χ^2 periodogram. For masking, the animals were kept on the ultradian 3.5/3.5-h light/dark cycle for 11 days. Data from the last 7 days, which covered light and dark periods spanning the entire circadian cycle, were used for calculating the percentages of wheel-running activity in darkness.

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A TRPV family ion channel required for hearing in *Drosophila*

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The many types of insect ear share a common sensory element, the chordotonal organ, in which sound-induced antennal or tympanal vibrations are transmitted to ciliated sensory neurons and transduced to receptor potentials[1,2]. However, the molecular identity of the transducing ion channels in chordotonal neurons, or in any auditory system, is still unknown[3,4]. *Drosophila* that are mutant for NOMPC, a transient receptor potential (TRP) superfamily ion channel, lack receptor potentials and currents in tactile bristles[5,6] but retain most of the antennal sound-evoked response[7], suggesting that a different channel is the primary transducer in chordotonal organs. Here we describe the *Drosophila* Nanchung (Nan) protein, an ion channel subunit similar to vanilloid-receptor-related (TRPV) channels of the TRP superfamily. Nan mediates hypo-osmotically activated calcium influx and cation currents in cultured cells. It is expressed *in vivo* exclusively in chordotonal neurons and is localized to their

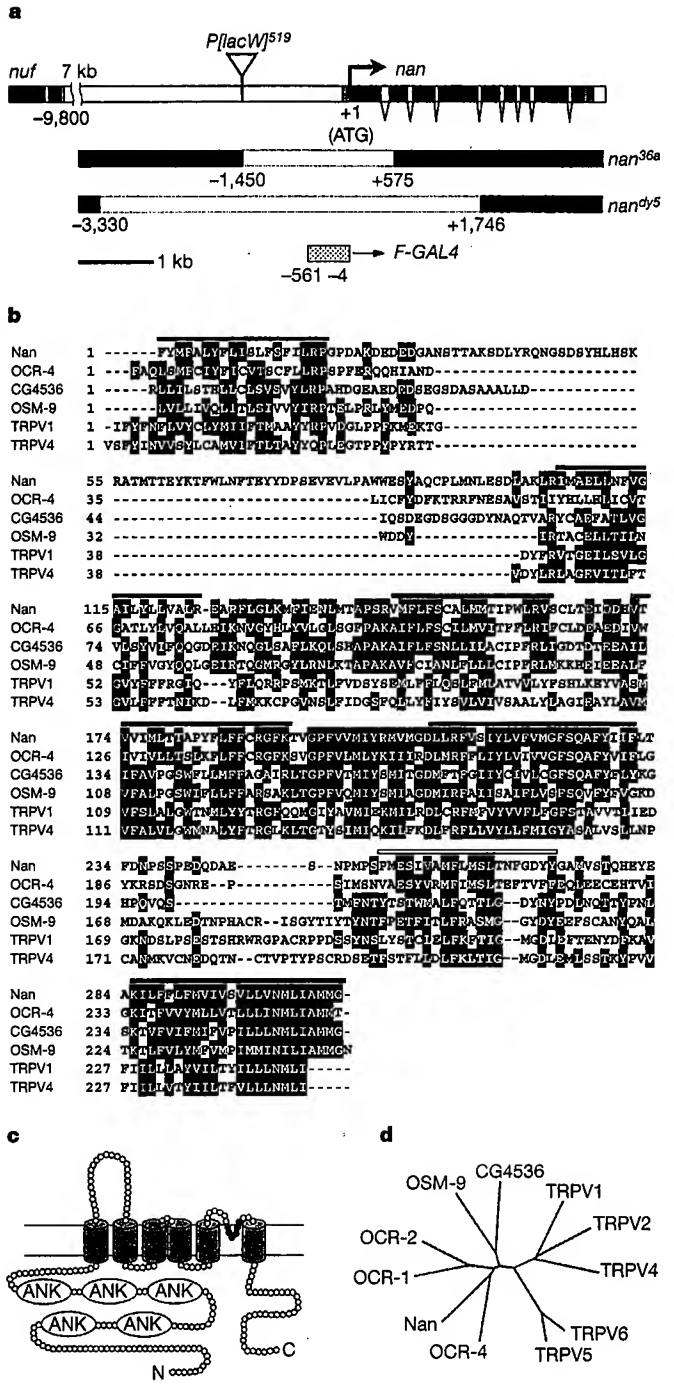

Figure 1 Molecular analysis of *nan*. **a**, Genomic organization. A transcription start site at −91 with respect to the translation start (+1) is indicated (angled arrow). A P transposon (*P[lacW]*^{519}) inserted 1.4-kb upstream of the transcription start site was imprecisely excised to give the deletions *nan*^{36a} and *nan*^{dy5}, deleting 150 and 478 codons, respectively. The *F-GAL4* construct contains a 557-bp fragment (dotted box). **b**, Alignment of the transmembrane region from TRPV proteins, including *Drosophila* Nan and CG4536, *C. elegans* OCR-4 and OSM-9, and human TRPV1 and TRPV4. Transmembrane domains (closed bars) and a putative pore region (open bar) were predicted by TMHMM2.0 (ref. 25). Black shading, identical amino acids; grey shading, similar amino acids; as used by Boxshade program (www.ch.embnet.org). **c**, Topology model for Nan protein, based on SMART[26] and TMHMM2.0 predictions. The putative pore region is denoted by a closed circle. **d**, Phylogenetic tree of the TRPV ion channel subfamily, based on a CLUSTALW alignment.